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Relationship between viability of thawed semen and pregnancy rate of Nelore cows subjected to fixed-time artificial insemination

Relação da viabilidade do sêmen criopreservado sobre a taxa de prenhez na inseminação artificial em tempo fixo de vacas Nelore

Relación de la viabilidad del semen criopreservado con la tasa de preñez en la inseminación artificial a tiempo fijo de vacas Nelore

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Abstract

This research aimed to evaluate the relationship of membrane integrity analysis, mitochondrial cytochemical analysis, and sperm kinetics of bovine semen after thawing and the pregnancy rate in Nelore females submitted to fixed-time artificial insemination (FTAI). Thirty-seven conventional semen straws, from 16 Nelore bulls and 3 Aberdeen Angus bulls were used. The semen samples were evaluated in conventional microscopy for sperm motility, vigor, and morphology, through flow cytometry for membrane integrity and mitochondrial function, and the CASA system for evaluation of sperm kinetics. All females were subjected to the same FTAI protocol and were inseminated by the same technical team. The correlation between all variables was performed by Pearson's correlation. Cluster analysis was used to analyze the relationship between pregnancy rate and combinations of laboratory tests ($P \leq 0.05$). Semen samples from Angus bulls (89.2 ± 5.5) presented superior VSL (P=0.004) to Nelore semen (78.6 ± 8.6), but this did not affect the pregnancy rate (Nelore: 51.0% versus Angus: 50.5%, $P > 0.05$). By flow cytometry, 44.3% membrane integrity and 47.3% mitochondrial integrity were identified. Five clusters were selected, one of which presented the best pregnancy rate, containing the most balanced rates between progressive velocity and mitochondrial activity (VSL

71.16 μm/s, MITO 49.69%, TxP 62.4%). Samples of thawed bovine semen with progressive velocity and balanced mitochondrial activity demonstrated better fertility to TAI program.
Keywords: Bovine; CASA system; Flow cytometry; Pregnancy.

Resumo
Objetivou-se avaliar a relação entre a análise de integridade de membrana, atividade mitocondrial e cinética de espermatozoides bovinos pós-descongelação, com a taxa de prenhez em fêmeas Nelore submetidas à inseminação artificial em tempo fixo (IATF). Foram utilizadas 37 doses de sêmen convencional, de 16 touros Nelore e 3 touros Aberdeen Angus. As amostras de sêmen foram avaliadas sob microscopia óptica para motilidade, vigor e morfologia espermática; a citometria de fluxo avaliou integridade de membrana e função mitocondrial; e o sistema CASA analisou a cinética espermática. As vacas foram submetidas ao mesmo protocolo da IATF e inseminadas pela mesma equipe técnica. A relação entre todas as variáveis foi feita por meio da correlação de Pearson. A análise de agrupamento foi usada para avaliar a relação entre a taxa de prenhez e as combinações de teste *in vitro* ($P \leq 0,05$). Amostras de sêmen de touros Angus (89,2 ± 5,5) apresentaram maior VSL ($P = 0,004$) do que sêmen de Nelore (78,6 ± 8,6), mas isso não afetou a taxa de prenhez (Nelore: 51,0% vs Angus: 50,5%, $P > 0,05$). Na citometria de fluxo identificou-se 44,3% de integridade de membrana e 47,3% de integridade mitocondrial. Cinco grupos foram selecionados, sendo que o cluster contendo valores mais equilibradas entre velocidade progressiva e atividade mitocondrial (VSL 71,16 μm / s, MITO 49,69%, TxP 62,4%) demonstraram maior taxa de prenhez. Amostras de sêmen bovino descongelado com taxa progressiva e atividade mitocondrial balanceada demonstraram fertilidade melhorada para o programa IATF.
Palavras-chave: Bovino;
; Prenhez; Sistema CASA.

Resumen
El objetivo fue evaluar la relación entre el análisis de la integridad de la membrana, la actividad mitocondrial y la cinética de los espermatozoides bovinos, con taxones de preñez en hembras Nellore sometidas a inseminación artificial por tiempo fijo (IATF). Muestras de semen fueron validadas por microscopía óptica para la motilidad, vigor y morfología de los espermatozoides; la citometría de flujo evaluó la integridad de la membrana y la función mitocondrial; y el sistema CASA analizó la cinética de los espermatozoides. Las vacas fueron sometidas al mismo protocolo IATF e inseminadas con el mismo equipo técnico. Una relación entre los diversos se encuentra a través de la correlación de Pearson. Un análisis de conglomerados evaluó la relación entre las tasas de preñez y combinaciones de pruebas *in vitro* ($P \leq 0,05$). Las señales de semen de toros Angus (89,2 ± 5,5) presentarán un VSL más alto ($P = 0,004$) que el semen de Nelore (78,6 ± 8,6), pero esto no afectó la tasa de preñez (Nelore: 51, 0% frente a Angus: 50,5%, $P > 0,05$). La citometría de flujo identificó 44,3% de integridad de membrana y 47,3% de integridad mitocondrial. Se seleccionaron cinco grupos, y el grupo que contiene valores más equilibrados entre la velocidad progresiva y la actividad mitocondrial (VSL 71,16 μm / s, MITO 49,69%, TxP 62,4%) demostrarán tasas de preñez más altas. Muestras de semen bovino con taxones progresivos y actividad mitocondrial equilibrada muestran una mejora de la fertilidad para el programa IATF.
Palabras clave: Bovino; Citometría de flujo; Preñez; Sistema CASA.

1. Introduction

Fixed-time artificial insemination (FTAI) is a biotechnique that occupies a prominent position as it allows AI of large numbers of females in a fixed-time, without the need for estrus observation, and so optimizes the reproductive management (Bó et al., 2018; Negreiros et al., 2020; Tortorella et al., 2016). Due to the evolution of FTAI in Brazil, in the last 15 years, semen commercialization has presented an expressive increase, with more than 12 million straws commercialized, almost double the number of straws sold in the year 2000, (ASBIA, 2015; P Baruselli et al., 2012; Meneghetti et al., 2009).

The Artificial insemination (AI) is a breeding biotechnology intended to providing greater genetic development and economic return for livestock (Fontes et al., 2020; Negreiros et al., 2020). However, there are reports of low pregnancy rates in AI animals, due to failures in estrus detection and a high incidence of postpartum anestrus.(Baruselli et al., 2018) In *Bos indicus* females, reproductive performance can be aggravated due to the high occurrence of nocturnal estrus and the short duration of estrus (Baruselli et al., 2004; Pinheiro et al., 1998).

Among the factors that interfere in the pregnancy rate of FTAI program in cattle, the body condition score, equine chorionic gonadotropin use, and semen quality are the most important (Marques et al., 2018; Negreiros et al., 2020). Conventional analyzes of frozen semen samples are subjective evaluations performed using optical microscopy. These

evaluations have little predictive value in relation to fertility, and do not offer a reliable parameter for subsequent fertility evaluation (Kathiravan et al., 2011). For this reason, computerized analyzes of sperm kinetics (Computer assisted semen analysis - CASA) are routinely used both commercially and in scientific projects for quality control and as a possible predictive method to evaluate the fertility of the bull before AI (Amann & Waberski, 2014).

In the proposal to evaluate a tool as a fertility predictor, the thermoresistance tests (TTR) were not considered effective to estimate the fertility of semen used in cows submitted to large-scale FTAI (Vianna et al., 2009). However, the association of TTR, with evaluation of spermatozoa kinetics by CASA, flow cytometry for plasma membrane integrity evaluation, and acrosome and mitochondrial functionality has been used as a relevant method for semen evaluation as these are fundamental structures for the transport and fertilization of spermatozoa (Oliveira et al., 2013).

In this context, there is a constant search for information that can predict sperm fertilization capacity, as well as the best associations between structural and functional laboratory tests which are highly correlated with field results. Thus, considering that few studies have been conducted to evaluate the relationships between semen quality and pregnancy rates in cows submitted to FTAI on a large scale, this study aimed to correlate the results of plasma membrane integrity analysis, the mitochondrial cytochemical activity of spermatozoa, and the sperm kinetics from post-thawed bovine semen with the pregnancy rate of Nelore females in FTAI program.

2. Methodology

2.1 Ethical aspects

The research was carried out according to the National Council of Control of Animal Experimentation (CONCEA) norms and approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Norte do Paraná (UNOPAR), registered under number 011 / 16.

2.2 Location and animals

The females were kept in a rural property, in the municipality of Brasilândia, Mato Grosso do Sul, located at latitude 21º15'21 "south and longitude 52 ° 02'13" west of Greenwich. The animals were maintained in flat pastures, cultivated with *Urochloa brizantha* grass. The utilization rate was approximately 1 AU / Ha.

A total of 4,171 Nelore multiparous females, aged between 3 and 10 years old, with 40 to 60 days postpartum, with a body condition score of 3.0 (1 to 5 scale) (Machado et al., 2008) were used, distributed in lots of approximately 150 animals, during a single mating season of five months. Water and mineral salt were supplied *ad libitum* throughout the experimental period.

2.3 Semen analysis

To the present study it was analyzed 37 straws of bovine frozen conventional semen were, 16 from Nelore bulls and 3 from Aberdeen Angus bulls, from different specialized centers. The semen straws were used according to the farm routine, in an FTAI program. For each departure, a straw was sent to the Laboratory of Biotechnology in Animal Reproduction, from Seleon Biotecnologia (Itatinga, SP, Brazil) for semen analysis.

2.4 Optical microscopy

After thawing the semen dose at 37ºC for 30 seconds, a 10µL aliquot of semen was subjectively analyzed for rectilinear sperm motility (expressed as a percentage from 0 to 100%) and sperm vigor (0 to 5 scale) under optical microscopy

with contrast of differential interference (BX 53, Olympus®, Tokyo, Japan) at increases of 100 and 200X according to CBRA (2013) recommendations.

For the morphological analysis of spermatozoa characteristics, an aliquot of semen was diluted in buffered saline solution (Hancock, 1959). Morphological analysis was performed with wet mount preparations using optical microscopy with differential phase interference contrast (BX 53, Olympus®, Tokyo, Japan) at a magnification of 1000X. The spermatic defects were computed using the classifications major, minor, and total defects (Blom, 1983).

2.5 Computerized evaluation (CASA)

The computerized analysis of the spermatic kinetics was carried out in a lamina propria with four chambers (LEJA®, Leja Products B.V., Nieuw-Vennep, The Netherlands). An aliquot of semen (20 µL) was diluted in sodium citrate solution (Na3C6H5O7) at 1: 1 dilution. Subsequently, the sample was transferred to IVOS® equipment, Hamilton Thorne, Beverly, MA, USA and analyzed by the software CASA II, Hamilton Thorne, Beverly, MA, USA, using the bovine setup (Hamilton Thorne, Beverly, MA, USA). Five homogeneous fields were selected by the responsible technician. The sperm kinetic parameters analyzed were total motility (%, proportion of moving cells), progressive motility (%, percentage of cells moving progressively), path velocity (VAP, µm/s, mean uninterrupted velocity of the cell path), linear velocity (VSL, µm/s, average speed traversed between the initial and final points of the trajectory), linearity (STR,%, mean value of VSL / VAP ratio), and linearity of the proportion between VSL / VCL (Arruda et al., 2010).

2.6 Flow cytometry

For the plasma membrane integrity evaluation, mitochondrial integrity and functionality, flow cytometry (Guava-Easycyte®, Merch Millipore, Darmstaldt, Germany) was used on a proprietary plate and specific fluorescent probes according to the manufacturer's recommendations.

For the membrane integrity evaluation (EASYKIT Viability Re. 024708 - IMV Technologies France), the probes propidium iodide (for sperm cells with damaged membrane) and sybr 14 (for sperm cells with whole membrane) were used in an 80-well plate. The staining was obtained using 1 µL of semen and 199 µL of buffer (supplied by the manufacturer) in each well. The plate was then incubated at 37 ° C for 10 minutes before being transferred to the flow cytometry equipment for reading.

For mitochondrial integrity and functionality (EASYKIT II Mitochondrial Activity Re. 024864 - IMV Technologies France) the JC-1 probe was used, also in an 80-well plate containing the probes. A 1 µL sample of semen was added to the plate together with 10 µL of absolute alcohol and 190 µL of buffer-phosphate buffer pH 7.0 (PBS). Next, the plate was incubated at 37ºC for 30 minutes and then transferred to the flow cytometry equipment, classified as polarized (active) and depolarized (inactive) mitochondria.

2.7 Fixed-time artificial insemination (FTAI) and pregnancy diagnosis

All females were submitted to the same FTAI protocol and were inseminated by the same technical team. The hormonal protocol included three treatments, started on a random day of the estrous cycle. On day 0 (D0), an intravaginal device containing 1g of progesterone (P4) (DIB®, Zoetis, São Paulo, Brazil) was applied, associated with the application of 2.0 mg of estradiol benzoate (Gonadiol®, Zoetis, São Paulo, Brazil), intramuscularly (IM). On day 8 (D8) the P4 device was removed and 250µg of sodium cloprostenol (Ciosin®, MSD Saúde Animal, São Paulo, Brazil) was administered; 1.0 mg of estradiol cypionate (EC) (ECP®, Zoetis, São Paulo, Brazil) and 300 IU of equine chorionic gonadotrophin (Folligon®, MSD

Animal Health, São Paulo, Brazil). Artificial insemination was performed on day 10 (D10), 48 hours after removal of the P4 device. Pregnancy diagnosis was performed by transrectal ultrasonography (Aloka SSD-500®, 5.0 MHz), 30 days after FTAI.

2.8 Statistical analysis

For the data analysis, the software SPSS, version 20.0 (IBM® Corporation, NY, USA) was used. Data from the semen analysis were submitted to the Kolmogorov-Smirnov test. The variables with normal distribution were submitted to the t-test and the non-parametric variables to the Mann and Whitney test. The pregnancy rate was defined by the ratio between pregnant cows and cows submitted to FTAI, compared by the chi-square test. Subsequently, a correlation between all variables was performed using a scatter diagram with the hypothesis test (Pearson's correlation). Cluster analysis was also used to find the relationship between pregnancy rate and combinations of laboratory analyzes. $P \leq 0.05$ was considered significant.

3. Results

The overall pregnancy rate to FTAI was 50.8% (2120/4171) and bull breed was not considered a source of variation (P>0.05) in the present study (Table 1).

Table 1. Comparison of pregnancy rates of Nelore cows submitted to FTAI with frozen semen of Nelore and Angus breeds.

Bull breed	N	Pregnancy rate (%)	P-value
Nelore	3451	51.0 (1760/3451)	P>0,05
Angus	720	50.0 (360/720)	
Total	4171	50.8 (2120/4171)	

Fonte: Autores.

Except for progressive velocity (VSL; P <0.05), all other parameters analyzed by optical microscopy and computerized analysis did not differ (P > 0.05) between Nelore and Angus bulls (Table 2). However, a lower total motility value (P = 0.023) was found in computerized analysis than in optical microscopy (Table 3).

Table 2. Values of the seminal analyzes, considering the Nelore and Angus breeds.

Genetic group	Parameters evaluated									
	MT* (%)	DEF* (%)	MEMB** (%)	MITO** (%)	MOT** (%)	MP** (%)	VAP** (µm/s)	VSL** (µm/s)	LIN** (µm/s)	STR** (µm/s)
Nelore	45.0 (30-70)	11.0 (1-25)	43.5 (±12.1)	46.5 (±9.1)	38.9 (±12.7)	27.8 (±9.6)	95.9 (±12)	78.6[a] (±8.6)	48.8 (±5.2)	81.2 (±4.2)
Angus	44.3 40-50)	15.0 (9-30)	48.2 (±9.2)	52.6 (±6.4)	40.7 (±10.2)	31.6 (±7.5)	103.9 (±6.2)	89.2[b] (±5.5)	51.5 (±2.3)	84.2 (±2.5)

Legend: MT = subjective motility in optical microscopy; DEF = spermatic morphology (% total defects); MEMB = plasma membrane integrity; MITH = mitochondrial activity; MOT = objective motility (CASA System); MP = progressive motility (CASA System); VAP = path velocity; VSL = progressive velocity; LIN = linearity; STR = straightness.
* Median (minimum and maximum) Mann-Whitney test
** Mean (± standard deviation) t-test
a ≠ b P = 0.004
Fonte: Autores.

Table 3. Comparison of total motility (median, minimum, and maximum values) analyzed by optical microscopy and computerized analysis of 37 frozen semen samples.

Method	n	Total motility (%)
Optical microscopy	37	45.0 (30 to 45)
Computerized analysis	37	39.3 (16.8 to 62.8)
P-value	-	0.023

Fonte: Autores.

Subsequently, when submitted to the Pearson's correlation test, no direct or individual correlations were found between the variables analyzed in the semen and the pregnancy rate (Table 4). However, there were correlations ($P < 0.05$) among sperm parameters, which a positive correlation was found between objective motility (MOT) and progressive motility (MP); path velocity (VAP) and progressive velocity (VSL); linearity (LIN) and rectilinearity (STR).

Table 4. Pearson correlation results (r), P-value, and number of semen straws (n) between results of semen laboratory tests and pregnancy rate.

Variables	DEF	MEMB	MITO	MOT	MP	VAP	VSL	LIN	STR	TxP
MT (r)	-	0.281	0.205	0.486**	0.532**	0.303	0.292	-	-0.091	0.051
(p)	0.011	0.093	0.286	0.002	0.001	0.069	0.080	0.194	0.593	0.765
(n)	0.951	37	29	37	37	37	37	0.249	37	37
	37							37		
DEF (r)	--	0.162	0.380*	-0.13	-0.065	0.154	0.113	-	-0.122	0.096
(p)		0.338	0.042	0.940	0.704	0.362	0.504	0.028	0.473	0.572
(n)		37	29	37	37	37	37	0.827	37	37
								37		
MEMB(r)	--	--	0.476**	0.504**	0.484**	0.187	0.137	-	-0.245	-
(p)			0.009	0.001	0.002	0.268	0.419	0.231	0.144	0.036
(n)			29	37	37	37	37	0.168	37	0.832
								37		37
MITO(r)	--	--	--	0.329	0.332	0.081	0.099	0.071	0.055	0.015
(p)				0.081	0.079	0.676	0.610	0.716	0.779	0.937
(n)				29	29	29	29	29	29	29
MOT(r)	--	--	--	--	0.940**	0.405*	0.305	-	-0.242	-
(p)					0.000	0.013	0.066	0.200	0.149	0.047
(n)					37	37	37	0.235	37	0.781
								37		37
MP(r)	--	--	--	--	--	0.381*	0.435**	0.028	0.027	-
(p)						0.020	0.007	0.871	0.872	0.033
(n)						37	37	37	37	0.848
										37

VAP(r)	--	--	--	--	--	--	0.869**	-	-0.378*	-
(p)							0.000	0.376	0.021	0.092
(n)							37	0.022	37	0.588
								37		37
VSL(r)	--	--	--	--	--	--	--	0.065	0.101	-
(p)								0.703	0.551	0.077
(n)								37	37	0.649
										37
LIN(r)	--	--	--	--	--	--	--	--	0.928**	0.067
(p)									0.000	0.693
(n)									37	37
STR(r)	--	--	--	--	--	--	--	--	--	0.113
(p)										0.508
(n)										37

Legend: MT = subjective motility in optical microscopy; DEF = spermatic morphology (% total defects); MEMB = plasma membrane integrity; MYTH = mitochondrial activity; MOT = objective motility (CASA); MP = progressive motility (CASA); VAP = path velocity; VSL = progressive velocity; LIN = linearity; STR = straightness; TxG = pregnancy rate.
Fonte: Autores.

The data were submitted to analysis of clusters with combinations between the *in vitro* laboratory analyzes and the pregnancy rate result, with only one combination presenting good cluster quality (Table 5). The grouping between VSL, MITO, and TxP was the one that presented the best result, with superior results (d> 0.5), in which 29 departures of semen were analyzed, as presented in Table 6.

Table 5. Five examples of the results from sperm evaluations and pregnancy rate by Cluster analysis.

Test	Optical microscopy	Flow cytometry	CASA	Pregnancy rate	Cluster quality
1	MT DEF	MEMB MITO	MOT MP VAP VSL LIN STR	TxP	Low (d ≥0)
2	MT DEF			TxP	Low (d ≥0)
3		MEMB MITO		TxP	Low (d ≥0)
4			MOT MP VAP VSL LIN STR		Low (d ≥0)
5		MITO	VSL	TxP	Good (d >0.5)

Legend: MT = subjective motility in optical microscopy; DEF = spermatic morphology (% total defects); MEMB = plasma membrane integrity; MITO = mitochondrial activity; MOT = objective motility (CASA); MP = progressive motility (CASA); VAP = path velocity; VSL = progressive velocity; LIN = linearity; STR = straightness; TxP = pregnancy rate.
Fonte: Autores.

Table 6. Representation of the best cluster result in the Cluster analysis.

Cluster	n (%)	VSL (μm/s)	MITO (%)	TxP (%)
Cluster 1	24.1	76.4	46.2	34.3
Cluster 2	13.8	82.3	32.0	50.2
Cluster 3	31.0	71.1	49.7	62.4
Cluster 4	31.0	91.2	53.4	53.6

Legend: n = percentage of semen departures; VSL = progressive velocity MITO= percentage of polarized mitochondria; TxP = FTAI pregnancy rate.
Fonte: Autores.

4. Discussion

In the present study, methods with direct relation to fertility were used to evaluate semen, such as kinetics, mitochondrial activity, and membrane integrity, as well as conventional analyzes of sperm morphology. To the best of our knowledge this is one of the few studies that combined the best *in vitro* laboratory analysis with pregnancy rate results using cluster analysis. These analyzes have significant importance for large-scale FTAI programs, since the establishment of predictive fertility analyzes, before the breeding season, is essential to obtain adequate pregnancy rates.

In Brazil, the minimum parameters for frozen bovine semen commercialization are established by the Brazilian College of Animal Reproduction (CBRA). Thus, the results obtained in the conventional evaluation of all thawed semen straws met the standards required by Colégio Brasileiro de Reprodução Animal (2013). Motility is considered one of the most important fertility traits. The total motility values evaluated in the present study, through optical microscopy, differed ($P = 0.023$) from the computerized analysis results. In fact, these findings have low predictive value for frozen semen fertility, corroborating previous results observed by other researchers (Kathiravan et al., 2011).

Although there was a difference ($P = 0.004$) in the progressive velocity (VSL) for semen of Angus bulls, there was no increase in the pregnancy rate in relation to cows inseminated with Nelore bulls (51.0% versus 50.0%, $P > 0.05$). Results from membrane integrity (43.5 for Nelore versus 48.2% Angus) and mitochondrial potential (46.5 for Nelore 52.6% for Angus) demonstrated that cryopreservation processing may have altered sperm cells, but sperm motility (38.9 to 40.7%) and pregnancy rate (50.8%) remained acceptable.

There was no correlation for any parameters obtained by the semen analyzes, used for FTAI, on the pregnancy rate. In the present study, no correlation was observed between sperm motility and mitochondrial function, different from the study with human spermatozoa, in which Troiano et al. (1998) found that the number of spermatozoa with depolarized mitochondria correlated positively with the percentage of immobile cells ($r = 0.52$, $p = 0.004$) and negatively with rapid progressive motility ($r = 0.55$, $p = 0.002$). The identification of mitochondrial potential was considered a good technique for ovine semen evaluation, having a correlation with the cell energetic potential and with sperm motility (Bergstein et al., 2014; Martinez-Pastor et al., 2004; Tsakmakidis, 2010). Some researchers have found variation in the correlation values between CASA results, mitochondrial function, membrane inefficiency and no return to estrus or pregnancy rate (Kathiravan et al., 2011; Rodríguez-Martínez, 2003). Multivariate regression analyzes such as Partial Least Squares (PLS) or a combination of data by Bayesian inference analysis have been utilized to better understand the associations of semen analysis results as predictive data on fertility (Hirao, 1975; Oliveira et al., 2013; Sellem et al., 2015; Sudano et al., 2011).

The present study performed a cluster formation to interpret results in relation to pregnancy rates. In the groups formed by the Cluster analysis, the spermatozoa labeled as good quality presented higher progressive velocity (VSL) and greater mitochondrial activity (MITO); however, were not those that provided the best pregnancy rates. On the other hand, in

Clusters 2, 3, and 5 pregnancy rates between 50.2 and 62.3% were observed, routinely found in Nelore cows submitted to FTAI. Cluster 5 presented the best pregnancy rate, which contained the most balanced parameter results, between progressive velocity and mitochondrial activity (VSL 71.16 μm/s, MITO 49.69%, TxP 62.4%). One hypothesis for this finding relates to mitochondria being the main source of pro-oxidative factors, suggesting that it has a central role in oxidative imbalance, and this organelle can have a positive and negative impact on the processes of fertilization.

Mitochondrial dysfunctions, caused by cryopreservation, are related to oxidative stress and decreased mitochondrial activity (O'Connell et al., 2002; Rui et al., 2015). These effects occur through the production of reactive oxygen species (ROS), which have a fundamental role in several physiological processes, such as sperm hyperactivation, sperm capacitation, acrosome reaction, and the interaction between spermatozoa and zona pellucida (Aitken et al., 2004; R.J. Aitken et al., 1995; de Lamirande & Cagnon, 1993; Lamirande et al., 1998).

Differently from AI after estrus observation, in FTAI, there is greater dispersion at the time of ovulation among synchronized females. The early sperm capacitation, associated with the oxidative stress and greater progressive velocity, could reduce spermatozoa survival in the female genital tract, consequently restricting the fertilization ability of these spermatozoa. The first stage of ROS formation is the generation of anion superoxide (Koppers et al., 2008), in this context, mitochondria seem to be the main factor responsible for imbalances between antioxidant mechanisms and ROS production, characterizing oxidative stress, which can be lethal to spermatozoa (Halliwell & Gutteridge, 1999), and may interfere with spermatozoa viability in the genital tract of females.

Prediction of *in vivo* fertility of thawed bovine semen by *in vitro* laboratory analysis remains a challenge for the semen production industry. The use of the Cluster analysis employed in the present study may provide an important way to interpret the laboratory results.

5. Conclusion

Based on the study conditions it can be concluded that the laboratory analyzes of thawed bovine semen alone is not sufficient to predict *in vivo* fertility. However, Cluster analysis revealed that samples of thawed bovine semen with progressive velocity and balanced mitochondrial activity presented better fertility rates.

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